

Supplementary Methods

Dosimetric characterization of pulsed irradiation

The dosimetric characterization of the irradiation beams was performed using the fD dosimetry system (*Marinelli et al., 2022*), employing different dosimeters and measurement techniques developed at CPFR as well as devices provided by collaborating institutions (*Ciarrocchi et al., 2024; Cova et al., 2025; Del Sarto et al., 2025; Di Martino, Del Sarto, Barone, et al., 2022; Di Martino, Del Sarto, Giuseppina Bisogni, et al., 2022; Di Martino et al., 2020; Gómez et al., 2022; Milluzzo et al., 2024; Romano et al., 2023*).

As reported in Table 1, FLASH irradiations were delivered using a dose-per-pulse (DPP) of 4.75 Gy with a pulse duration of 4 μ s. By varying the pulse repetition frequency (PRF), it was possible to maintain a constant average dose rate (ADR) of 940 Gy/s for both total doses investigated in this study. This value largely exceeds the minimum threshold generally reported to induce the FLASH effect (100 Gy/s) and can be preserved independently of the delivered total dose. For a pulsed beam consisting of n pulses, the ADR—neglecting the finite pulse duration—can be expressed as:

$$ADR = \frac{n}{n - 1} \cdot DPP \cdot PRF$$

The dose values reported in Table 1 refer to the build-up region, corresponding to the point of maximum dose along the percentage depth dose (PDD) curve, as shown in **Additional File 2: Fig. S1a**.

The tumor volume had a finite depth extension ranging from the surface (0 mm) to approximately 5 mm, which lies within the plateau region of the PDD where the dose remains above 95% of the maximum value. Moreover, the lateral beam profiles demonstrate dose homogeneity over a 7 mm diameter region (**Additional File 2: Fig. S1b**), within which the tumor masses of all animals were centered. The average dose delivered to the tumor was estimated by averaging tumor volumes along the PDD curve and exceeded 95% of the dose at the build-up point.

For healthy tissues, the estimated dose to the skin (first 1 mm depth) was approximately 96% of the build-up dose, while the dose delivered to the underlying muscle tissue (1–6 mm depth) was over 97% (**Additional File 2: Fig. S1a**).

Ultrasound imaging of tumor volumes

Ultrasound imaging was performed using a Vevo 3100 system (FUJIFILM VisualSonics Inc., Toronto, Canada) while the animals were under isoflurane anesthesia (induction: 2.5%, maintenance: 1.5%; flow 1 L/min). The left hindlimbs were shaved with depilatory cream, and the animals were secured on a heated stand. Temperature, respiration, and heart rate were monitored during the imaging process (THM-100; Indus Instruments, Houston, USA). A MS550 probe (22-55 MHz) was coupled to the mice with acoustic gel and used for all scans. B-mode and Power Doppler-mode images of the hindlimb tumor were acquired in short- and long-axis views with the animals in ventral recumbency, properly aligned to the target. Additionally, 3D-mode scans of the melanoma tumor were obtained at 70-150 μ m intervals.

Assessment of Skin Toxicity

Animals were sedated with isoflurane (1%, 1 L/min) and placed on a temperature-controlled board set at 37 °C. Animals were positioned in both supine and prone orientations, with the left hind limb gently extended to allow standardized image acquisition of the anterior and posterior aspects of the paw. At least four high-quality images were acquired per animal. Images were independently evaluated by at least three investigators blinded to the experimental groups using a semi-quantitative skin toxicity scoring system developed in this laboratory and adapted from previously described radiation-induced skin injury scales (*Kumar et al., 2008; Soto et al., 2020*), as illustrated in **Fig. 2b**. The scale ranged from 0 (normal skin) to 6 (severe damage), encompassing progressive manifestations from depigmentation and alopecia to dry and moist desquamation, ulceration, extension of injury beyond the irradiated field,

and muscle atrophy/shortening. Individual scores were averaged to obtain a final skin toxicity score for each animal.

RNA Isolation, cDNA synthesis and quantitative PCR

Total RNA was extracted from 10–15 mg of frozen mouse skin and muscle tissues, collected from the hindlimb of both control and irradiated mice. After homogenization in TRIzol reagent, samples were processed for the isolation of RNA. The aqueous phase obtained after chloroform addition and centrifugation ($12000 \times g$ for 15 min at 4 °C) was loaded onto Direct-zol RNA Microprep columns (Zymo Research, # R2060) and RNA eluted in 15 μ L RNase-free water, as indicated by the manufacturer protocol. cDNA was synthesized using iScript Reverse Transcription Supermix for RT-qPCR (#1708840, Bio-Rad Laboratories), and quantitative real-time PCR (qPCR) was carried out using either probe-based (iTaQ Universal Probes Supermix, Bio-Rad Laboratories) or SYBR Green-based (iTaQ Universal SYBR Green Supermix, Bio-Rad Laboratories) systems with CFX96™ Real-Time instrument (Bio-Rad Laboratories). Probes were acquired from Applied Biosystems and exon junction-spanning primers were designed using PrimerBLAST and acquired from Eurofins. Details are displayed in **Additional File 3: Table S2**. The relative amount of mRNA for CCL2, IL1b, Krt6a, Krt6b, ADAM8, BCL3, CDKN1A/p21 and PAI1/serpine1 genes was calculated as fold increase on TATA Binding Protein gene (TBP) expression (internal control). Data were obtained using $\Delta\Delta C_t$ method.

Protein Isolation and Western Blot Analysis

Total proteins were extracted from 22–25 mg of frozen hindlimb muscle tissue (vastus lateralis) of control and irradiated mice. After homogenization, protein lysates were cleared by centrifugation ($10,000 \times g$, 10 min, 4°C) and concentrations were determined by Bradford Assay (#5000001, Bio-Rad Laboratories) according to the manufacturer's instructions. Equal amounts of protein (30 μ g) were separated by SDS-PAGE on 4–15% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (#4568083, Bio-Rad Laboratories) and transferred to nitrocellulose membranes using the Trans-Blot® Turbo™ Transfer

System (#1704159, Bio-Rad Laboratories). Membranes were blocked with EveryBlot™ Blocking Buffer (#12010020, Bio-Rad Laboratories) for 30 min at room temperature, then incubated overnight at 4°C with the following primary antibodies from Cell Signaling Technology in blocking buffer: phospho-S6 (#5364, 1:1000), S6 (#2317, 1:1000), phospho-AKT (#4058, 1:1000), AKT (#9272, 1:1000), phospho-STAT3 (#9145, 1:1000), STAT3 (#9139, 1:500) and GAPDH (#2118, 1:3000). After washing with TBS-T (Tris-buffered saline containing 0.1% Tween-20), membranes were incubated with HRP-conjugated secondary antibodies from Bio-Rad Laboratories: Goat Anti-Rabbit IgG (H+L)-HRP (#1706515, 1:3000) and Goat Anti-Mouse IgG (H+L)-HRP (#1706516, 1:3000). Immunoreactive bands were detected using an enhanced chemiluminescence system (Clarity™ Western ECL Substrate, #1705060, Bio-Rad Laboratories). Densitometric analysis of the bands was performed using ImageJ software, and protein expression levels were normalized to GAPDH.

RNA Sequencing

RNA evaluation - Qualitative and quantitative RNA evaluations were performed by NanoDrop ND-1000 Spectrophotometer (ThermoFisher). To determine the integrity of RNA samples, check controls were performed by Agilent Bioanalyzer using RNA 6000 LabChip ® kit (#5067-1511) or Agilent Fragment Analyzer 5300 using RNA (15nt) kit (#5191-6572). All the samples with a RIN below 7.5 were discarded while the others were processed for libraries preparation and sequencing.

Library preparation - Total RNA samples (200 ng) were processed using the Illumina Stranded Total RNA Prep with Ribo-Zero Plus kit (#20040529). Ribosomal RNA (rRNA) was depleted following the manufacturer's *Sample Preparation Guide* (Document #1000000124514, version 03). Purified RNA was chemically fragmented using divalent cations under high temperature in Illumina proprietary fragmentation buffer. First strand cDNA was synthesized using random oligonucleotides and Reverse Transcriptase Enzyme and second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H.

After purification with Agencourt AMPure XP beads (#A63882, Beckman), DNA fragments were adenylated in their 3' ends, then Illumina–RNA Index Anchor (#20040899) adapters were ligated.

Library enrichment and indexing were carried out with Illumina RNA Unique Dual Indexes (#20091655) and PCR Mastermix in a 13 cycles amplification reaction. Finally, library products were purified with AMPure XP beads, quantified using both Tecan Infinite F200-PRO instrument and Qubit dsDNA BR reagent (#Q32853, ThermoFisher) and checked for quality on the Agilent 2100 Bioanalyzer using Agilent DNA 1000 assay (#5067-1504) or Agilent Fragment Analyzer 5300 and Double Strand DNA 910/915 (#5191-6595).

Individual indexed libraries were pooled to achieve equimolar concentrations of each sample. Specifically, 50 ng of library for each sample were collected in a vial, then the final pool was quantified using the Qubit dsDNA BR kit and the molarity was calculated considering an average library size of 340 bp.

Sequencing on NovaSeq X Plus - Pooled libraries were loaded and sequenced on Illumina NovaSeq X Plus using 1.5B Reagent Kit (300 Cycles) (#20104705) at 150pM final loading concentration. At the end of the run, around 140M of 151bp paired-end reads per sample were used for the bioinformatic analysis.

Bioinformatic analysis

Demultiplexing - On-board DRAGEN BCL Convert tool was used to demultiplex samples, generate individual datasets in the form of separate FASTQ files for reads 1 and 2, and to trim adapter sequences.

Single sample pipeline - The overall bioinformatic pipeline run for the samples is based on RiDE (Rossano Atzeni et al., n.d.), part of the solid-core workflow collection, developed at CRS4. The pipeline performs alignment, quantification, and quality controls.

Alignment - Reads in FASTQ format were aligned with STAR 2.5.3a (Dobin et al., 2013) using mouse reference UCSC Genome Browser mm10 (Kent et al., 2002), [mm10 mouse reference:

<https://genome.ucsc.edu/cgi-bin/hgGateway?db=mm10>]. As supporting gene track, the UCSC GenomeBrowser knownGene in GTF format was downloaded and adapted to the project aim.

Quantification - Transcript abundance was estimated with Kallisto 0.43.0 (Bray et al., 2016), which identifies the transcript sequences using a FASTA file representing known cDNA sequences. The cDNA sequences used have been obtained from Ensembl Genome Browser, with HGNC gene symbols.

Quality Control - Quality control metrics were obtained for each sample with the software RSeQC (Wang et al., 2012), version 5.0.3.

Differential Expression analysis- Differentially expressed (DE) genes were identified using the R package DeSeq2 (Love et al., 2014) using a FDR corrected p-value<0.05. In house scripts have been developed to evaluate the appropriate fold-change (FC) threshold and balance between p-value and FC.

Enrichment analysis - Functional enrichment analysis of differentially expressed genes was performed using the DAVID Bioinformatics Resources (Database for Annotation, Visualization and Integrated Discovery) (Huang et al., 2009). Separate lists of upregulated and downregulated genes, annotated with official gene symbols, were uploaded with *Mus musculus* selected as the background species. Enrichment analysis was conducted specifically for Gene Ontology Biological Process (BP - direct) terms and KEGG pathways. Annotation terms containing at least two genes and with a p-value<0.05 were considered significant.

Light microscopy

Sample preparation - tissue samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 24 h, progressively dehydrated in increasing concentrations of alcohol, clarified in xylene and finally embedded in paraffin as described by Castorina et al. (Castorina et al., 2021). From each sample, sections 3 µm thick were cut on a sliding microtome (Leica RM 2135, Leica Microsystem) and stained with haematoxylin and eosin (H&E) for routine use.

Immunohistochemistry - For immunohistochemical analysis, paraffin-embedded tissue sections were processed using the BenchMark ULTRA system (Roche Tissue Diagnostics), following the

manufacturer's instructions. The automated procedure included antigen retrieval with Ventana Ultra CC1 buffer (Roche Tissue Diagnostics), followed by incubation with the CONFIRM anti-CD68 (KP-1) primary antibody (Roche Tissue Diagnostics). Detection was performed using the ultraView Universal DAB Detection Kit (Roche Tissue Diagnostics). Finally, hematoxylin counterstaining and mounting were performed.

Histological Imaging and Quantitative Analysis - Tissue sections were examined using a light microscope (Zeiss Axioskop 40; Carl Zeiss GmbH), and digital images were captured with a Zeiss Axiocam 503 color camera for subsequent analysis of epidermal and dermal thickness, as well as CD68 protein expression. To assess tissue thickness, at least four fields of well-preserved tissue were randomly selected from each sample and imaged at $\times 2.5$ magnification. Epidermal thickness was measured from the surface epithelium to the top of the dermal papillae, while dermal thickness was measured from the base of the dermal papillae to the underlying dermal white adipose tissue. For CD68 expression analysis, at least four fields of well-preserved tissue were randomly selected from each sample and imaged at $\times 20$ magnification. Semi-automated image analysis was performed using ImageJ software. Images were converted to grayscale, and red-stained areas (indicating CD68 positivity) were selected using the threshold tool. CD68 expression was quantified as the percentage of CD68-positive area relative to the total selected tissue area.

Semithin and Transmission Electron Microscopy

Murine skin and muscle samples, approximately 1 mm^2 in size, were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for a minimum of 2 hours. Samples were then washed with 0.1 M sodium cacodylate buffer (pH 7.4) to remove glutaraldehyde and post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences) for 1 hour at room temperature. After rinsing with 0.1 M sodium cacodylate buffer (pH 7.4), samples were dehydrated through a graded acetone series (40%, 60%, and 100%) and infiltrated with resin/acetone

mixtures at ratios of 1:1 and 2:1, followed by embedding in pure epoxy resin (Electron Microscopy Sciences) and polymerization at 60 °C for 24 hours.

Semithin sections (800 nm) were obtained using a Powertome X ultramicrotome (RCM Boeckeler) equipped with a 2.5 mm diamond knife (Diatome) and stained on glass slides with 1% toluidine blue and 1% sodium tetraborate (Electron Microscopy Sciences). The stained sections were scanned using a NanoZoomer-SQ (Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with NDP.view2 software (Hamamatsu Photonics).

Ultrathin sections (~80 nm) were obtained using an 8 mm diamond knife (Diatome) and collected on 200-mesh copper grids (Electron Microscopy Sciences). Sections were counterstained with UranylLess (Electron Microscopy Sciences), followed by lead citrate (Electron Microscopy Sciences). Transmission electron microscopy (TEM) analysis was performed using a JEOL JAM-1400 Flash microscope operating at 120 kV and equipped with a high-sensitivity sCMOS camera (JEOL, Tokyo, Japan). Morphometric analysis of murine muscle myofibril thickness at both M-line and Z-line level was carried out using JEOL SightX Viewer 2.1 software (JEOL).

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